# Occurrence and characterization of Shiga toxin-producing *Escherichia coli* in raw meat, raw milk and street vended juices in Bangladesh

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**Running title: Shiga toxin-producing *Escherichia coli* in Bangladesh**

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## Abstract

The major objective of this study was to investigate the prevalence of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) in different types of food samples and to compare their genetic relatedness with STEC strains isolated previously from animal sources in Bangladesh. We investigated a total of 213 food samples, including 90 raw meat samples collected from retail butcher shops, 20 raw milk samples from domestic cattle, and 103 fresh juice samples from street vendors in Dhaka city. We found that more than 68% ($n$ = 62) of the raw meat samples were positive for the *stx* gene(s); 34% ($n$ = 21) of buffalo meats and 66% ($n$ = 41) of beef. Approximately 10% ($n$ = 2) of the raw milk and 8% ($n$ = 8) of the fresh juice samples were positive for *stx*. We isolated STEC O157 from seven meat samples (7.8%) of which two were from buffalo meats and five from beef, and no other STEC serotypes could be isolated. We could not isolate STEC from any of the *stx*-positive raw milk and juice samples. The STEC O157 isolates from raw meats were positive for the $stx_{2,}$ *eae*, *katP*, *etpD* and enterohemorrhagic *E. coli hly* ($hly_{EHEC}$) virulence genes, and belonged to three different phage types: 8 (14.3%), 31 (42.8%), and 32 (42.8%). Pulsed-field gel electrophoresis (PFGE) typing revealed six distinct patterns among seven isolates of STEC O157 suggesting a heterogeneous clonal diversity. Of the six PFGE patterns, one was identical and other two were ≥90% related to PFGE patterns of STEC O157 strains isolated previously from animal feces indicating that raw meats are readily contaminated with fecal materials. This study represents the first survey of STEC in the food chain in Bangladesh.



*Key words:* Shiga toxin-producing *Escherichia coli*, raw meat, raw milk, street vended juice

## Introduction

Shiga toxin-producing *Escherichia coli* (STEC) organisms, also called verocytotoxin (VT)-producing *E. coli* (VTEC), are one of the most important groups of foodborne pathogens (Karmali, 1989; Paton and Paton, 1998). They are a major cause of gastroenteritis that may be complicated by hemorrhagic colitis (HC) or the hemolytic-uremic syndrome (HUS), which is the main cause of acute renal failure in children. STEC are a serologically diverse group of zoonotic pathogens. Serotype O157:H7 has been reported as the predominant type worldwide (Tarr et al., 2005). Since its identification as a pathogen in 1982, STEC O157:H7 has been the cause of a series of outbreaks in Canada, Japan, the United Kingdom, the United States and other countries (Karmali, 1989; Paton and Paton, 1998; Willshaw et al., 2001). However, more than 200 STEC serotypes have been reported and more than 100 have been linked with human infection (Eklund et al., 2001).

The pathogenicity of STEC strains is associated with various virulence factors. The main factor is the capacity to form two potent phage-encoded cytotoxins called Shiga toxins (Stx1, Stx2 and their variants) or verocytotoxins (VT1 and VT2) (Paton and Paton, 1998). Another virulence factor associated with clinical STEC isolates is the protein intimin, encoded by the *eae* gene and responsible for the intimate attachment of STEC strains to the intestinal epithelial cells and causing the formation of attaching and effacing (A/E) lesions in the intestinal mucosa (Kaper et al., 1998). Enterohemolysin, also called enterohemorrhagic *E. coli* (EHEC) hemolysin has also been identified as a possible STEC virulence factor (Beutin et al., 1989).

STEC represent the only pathogenic group of *E. coli* that has a definite zoonotic origin, although not all the STEC strains have been demonstrated to cause disease in humans. STEC strains rarely cause disease in animals, and ruminants are recognized as their main natural

reservoir. Cattle are considered to be the major animal source of STEC that are virulent to humans, in particular STEC O157 (Caprioli et al., 2005). STEC O157 and other serotypes associated with human infections have also frequently been isolated from the intestinal content of other ruminant species, including sheep, goat, water buffalo, and wild ruminants. Contamination of carcasses with STEC usually occurs during slaughter and subsequent processing through fecal material originating directly or indirectly from the rectal-cecal area (Erickson and Doyle, 2007). Dairy products (milk, cheese, cream) associated with infection have included those that are unpasteurized, have had a pasteurization failure or have been contaminated post-pasteurization. Ready-to-eat foods have also been associated with infection, particularly cooked meats contaminated by raw materials during processing, in catering establishments, at retail sale, and in the home. In developed countries, STEC O157 outbreaks associated with beef have caused concern among public health workers, clinicians, and the public, prompting major changes in clinical and laboratory practice, meat production, and food preparation (Mahon et al., 1997).

Like many of the developing and underdeveloped countries, the hygienic conditions in Bangladesh are severely compromised, and living with domestic animals within the same premises is a common practice among Bangladeshi population. In our previous study we found that the prevalence of STEC in slaughter animals was 24% and 43% of these STEC isolates belonged to serogroup O157 (Islam et al., 2008). Although this high prevalence of STEC in animal reservoirs has little impact on public health considering the lack of STEC associated infections among Bangladeshi population (Islam et al., 2007), it is important to know the occurrence and transmission of these organisms in the human food chain.

Since the incidence of STEC in Bangladeshi foods has remained unknown, the study being reported here examined the occurrence of STEC in different types of food sample, with the isolates further characterized according to serotype, phage type, virulence gene profile, and

pulsed-field gel electrophoresis (PFGE) type. In addition, we compared the PFGE types of the food isolates with that of a collection of STEC strains isolated at adjacent period from animal feces in Bangladesh to find out clonal relatedness.

## Materials and methods

### *Sampling*

We collected randomly 90 raw meat samples (60 beef and 30 buffalo) from July 2006 to January 2007 from 15 retail butcher shops located in five different areas (3 from each area) in Dhaka city. From each butcher shop, we collected 6 meat samples at two different time points (3 at one time point) during the sampling period. In the same period, we randomly collected raw milk samples from 20 dairy cows domesticated at 20 different households located in two suburbs (10 from each area) of Dhaka city. In addition, we collected 103 freshly prepared juice samples, including mixed herbal juice ($n$ = 49), sugar cane juice ($n$ = 40), wood apple juice ($n$ = 7) and some other types of juice (three milk shake, one lemon, two mix fruit, and one pineapple) from street vendors who operate in the main streets of 10 different locations in the Dhaka city. Only one juice sample was collected at one time point from each vendor. Each sample (minimum of 25 g or 25 ml) was aseptically transferred to a sterile plastic bag, placed in an ice box, and transported to the laboratory within 2 h. We started microbiological investigation of the samples within 4 h of collection.

### *Isolation of STEC O157*

For meat samples, a 25-g portion, and for raw milk and juice samples, a 25-ml portion was added to 225 ml of modified tryptone soy broth (Oxoid Ltd., Basingstoke, United Kingdom). After homogenization in a stomacher for 1 min, the samples were incubated for 18-20 h at 37°C. STEC O157 strains were isolated as described previously (Islam et al.,

2006), by using the immunomagnetic separation (IMS) technique and presumptive isolates were confirmed by an agglutination test using *E. coli* O157 latex agglutination kit (Murex Biotech Ltd., Dartford, Kent, United Kingdom) and a PCR for the *rfb* (O-antigen-encoding) region of *E. coli* O157 (*rfb*$E_{O157}$) (Paton and Paton, 1998).

*Isolation procedure for STEC non-O157*

The overnight enrichment cultures were screened by PCR for the presence of *stx*$_1$ and *stx*$_2$ using the primers as described earlier (Islam et al., 2007). DNA was extracted from 1 ml of broth culture by thermal cell lysis using Chelex-100 resin (Bio-Rad Laboratories Ltd., Richmond, Calif.) and 5 µl of the DNA extract was used in the PCR (Malorny et al., 2003). Multiplex PCR for *stx*$_1$ and *stx*$_2$ was carried out in a PTC-200 peltier thermal cycler (Bio-Rad) using the program as described previously (Islam et al., 2007). DNA extracted from *E. coli* O157:H7 strain NCTC 12079 and *E. coli* strain ATCC 25922 were used as positive and negative controls, respectively in each PCR reaction. Broth cultures that yielded positive PCR results for *stx*$_1$ and/or *stx*$_2$ were streaked onto tryptone bile x-glucuronide (TBX) agar (Oxoid) supplemented with cefixime (20 mg/l), cefsulodin (3 mg/l) and vancomycin (30 mg/l) (CCV-TBX). Single colonies, including different morphological types were transferred from the CCV-TBX and plated onto Luria agar (Difco, BD diagnostics, USA) to create a grid pattern of 96 colonies (12×8). The plates were incubated overnight at 37°C. The isolates were subjected to PCR for the detection of *stx*$_1$ and *stx*$_2$ as described above. The number of PCR reactions was reduced to 20 by pooling the colonies per row and per column in 500 µl sterile distilled water. DNA was extracted by boiling the suspensions for 10 min and 5 µl of the supernatant was used in the PCR. The isolates with positive PCR results were identified as *E. coli* by an API 20E test (bioMérieux, Marcy l'Etoile, France).

*PCR detection of virulence factors*

PCR for detecting both chromosome (e.g., *stx*$_1$, *stx*$_2$, and *eae*)- and plasmid (e.g., *hly*$_{EHEC}$, *katP*, and *etpD*)-encoded virulence genes was performed as described earlier (Islam et al., 2007) in a total volume of 25 µl containing 2.5 µl of 10× Fast Start buffer with 1.5 mM $MgCl_2$ (Applied Biosystems, Weiterstadt, Germany), 200 µM concentration of each deoxynucleoside triphosphate, a 0.2 pmol of each primer, and 1 U of Fast start *Taq* DNA polymerase (Roche, Mannheim, Germany) using a PTC-200 peltier thermal cycler (Bio-Rad).

*Phage typing*

Isolates confirmed to be STEC O157 were phage typed at the laboratory of Enteric Pathogens, Central Public Health Laboratory, London, United Kingdom.

*PFGE*

PFGE was performed following the standardized protocol developed by PulseNet for *E. coli* O157:H7 (Ribot et al., 2006). Analysis of the TIFF images was carried out by the BioNumerics software (Applied Maths, Belgium) using the dice coefficient and unweighted-pair group method using average linkages to generate dendrograms with 1.0% tolerance values. Normalized PFGE patterns (bundle files) of the food isolates were compared with that of the animal isolates from our previous studies (Islam et al., 2008) in order to find out the clonal relationships.

## Results and discussion

*Occurrence of STEC in food samples*

Of the 90 meat samples tested in this study, we found that 62 (69%) were positive for *stx* gene(s); 21 samples of buffalo meats, and 41 samples of beef (Table 1). STEC O157 strains

were isolated from seven samples (7.8%); two samples of buffalo meats, and five samples of beef (Table 1). No STEC non-O157 strains were isolated. During the processing of carcasses, meat can easily become contaminated with animal feces if there are unhygienic conditions, lack of good processing practices and a lack of knowledge of the butchers on basic hygiene. These features are commonly observed in butcher shops in Bangladesh where the same premise is being used to slaughter the animal, to process carcasses and as vending point. Our result correlates with data reported from neighboring countries, including India where STEC O157 have been identified as one of the predominant serogroups from buffalo meats (Hazarika et al., 2007). STEC O157 strains isolated from raw minced beef samples (9%; $n$ = 22) (Dutta et al., 2000), beef surface swabs (3.7%; $n$ = 27) and milk samples (2.4%; $n$ = 81) (Manna et al., 2006) in India. Of the 20 raw milk samples, two (10%) were positive for *stx* gene(s) (Table 1). Of the 103 fresh juice samples, eight (8%) were positive *stx* gene(s); six samples of mixed herbal juices, and two samples of wood apple juices (Table 1). However, no STEC could be isolated from *stx*-positive samples of raw milk and street vended juices. This might be due to the presence of a very small number of organisms present in the sample that were insensitive to the culture method used in the study. Another reason might be the presence of free *stx* phages in the sample that turned out to be positive by PCR for *stx* genes.

*Characterization of STEC O157 isolates*

STEC O157 strains that were isolated from raw meat samples were further characterized. All isolates were positive for the $stx_2$ gene only. There is considerable epidemiological evidence to indicate that STEC O157 isolates producing Stx2 are more commonly associated with serious disease than isolates producing Stx1or Stx1and Stx2 (Law, 2000). In addition to $stx_2$ gene, all isolates in the study were positive for *eae*, *etpD, katP* and $hly_{\text{EHEC}}$ genes, which accentuate their capability to cause human infection.

The strains could be grouped into three different phage types (PT): PT 31 ($n$ = 4), PT 32 ($n$ = 2), and PT 8 ($n$ = 1). Phage types 31 and 32 have been commonly found among STEC O157 isolates obtained from both human and non-human sources (food and animal feces) from different countries suggesting a foodborne transmission of the organism (Khakria et al., 1990, Mannix et al., 2007). In our previous study we found that these two phage types (PT 31 and PT 32) were the predominant PTs among STEC O157 strains isolated from animal feces (Islam et al., 2008). Phage type 8 has been found frequently among STEC O157 strains from humans and bovine in many European countries, including Spain, Belgium, Finland, Germany, Italy, England and Scotland (Khakria et al., 1990). We also found this phage type in our previous study in a STEC O157 strain isolated from goat feces. Therefore, it is more likely that raw meat products are often contaminated with animal feces in the butcher shop environment.

PFGE analysis of the seven STEC O157 isolates revealed six distinct patterns, suggesting a genetically heterogeneous group. We compared these patterns with a panel of PFGE pattern that comprises 44 STEC O157 strains isolated previously from feces of cow, buffalo and goats from slaughterhouses in Dhaka city (Fig. 1). One strain (CM52) isolated from beef had an identical PFGE pattern with that of a strain isolated from buffalo feces (M139). Interestingly, both the beef and fecal strains were isolated from the same slaughterhouse (data not shown). Another beef isolate (CM2) had a PFGE pattern that was 92% related to that of a cluster of strains isolated from cow (C8) and goat feces (G72 and G85). Two strains, one from beef (CM56) and one from buffalo meat (MM28) belonged to the same clonal type (Fig. 1) and this PFGE pattern was 90% related to that of a strain isolated previously from cow feces (C21). Among the six PFGE patterns obtained from meat isolates, 3 (50%) were identical/closely (≥90%) related to that of the isolates from animal feces. However, this result was not concordant with the phage typing results. Overall results of the study indicate that

meats are readily contaminated with fecal materials containing STEC O157 in the butcher shops due to unhygienic practices of carcass processing.

## Conclusion

To our knowledge, this is the first survey to establish the prevalence of Shiga toxin-producing *E. coli* in various food items in Bangladesh. Despite frequent contamination of raw meat products by potentially virulent *E. coli* O157, to date, no clinical data about impact on human health are available in Bangladesh. Improved surveillance and hygienic conditions along with the development of control measures are therefore highly recommended to minimize contamination of raw meat products by potentially virulent *E. coli* O157.

## Acknowledgments

This research was funded by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) and by the Netherlands Foundation for the Advancement of Tropical Research (NWO-WOTRO) grant (Award number WB 93-415). ICDDR,B acknowledges with gratitude the commitment of the Netherlands Foundation for the Advancement of Tropical Research (NWO-WOTRO) grant to the Centre's research efforts. ICDDR,B also gratefully acknowledges these donors who provide unrestricted support to the Centre's research efforts: Australian International Development Agency (AusAID), Government of Bangladesh, Canadian International Development Agency (CIDA), The Kingdom of Saudi Arabia (KSA), Government of the Netherlands, Government of Sri Lanka, Swedish International Development Cooperative Agency (SIDA), Swiss Development Cooperation (SDC) and Department for International Development, UK (DFID).

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TABLE 1. PCR DETECTION OF *STX* GENE(S) IN BROTH CULTURES FROM FOOD SAMPLES AND ISOLATION OF SHIGA TOXIN-PRODUCING *E. COLI* O157 AND NON-O157 BY SUBCULTURING ONTO SELECTIVE AGAR

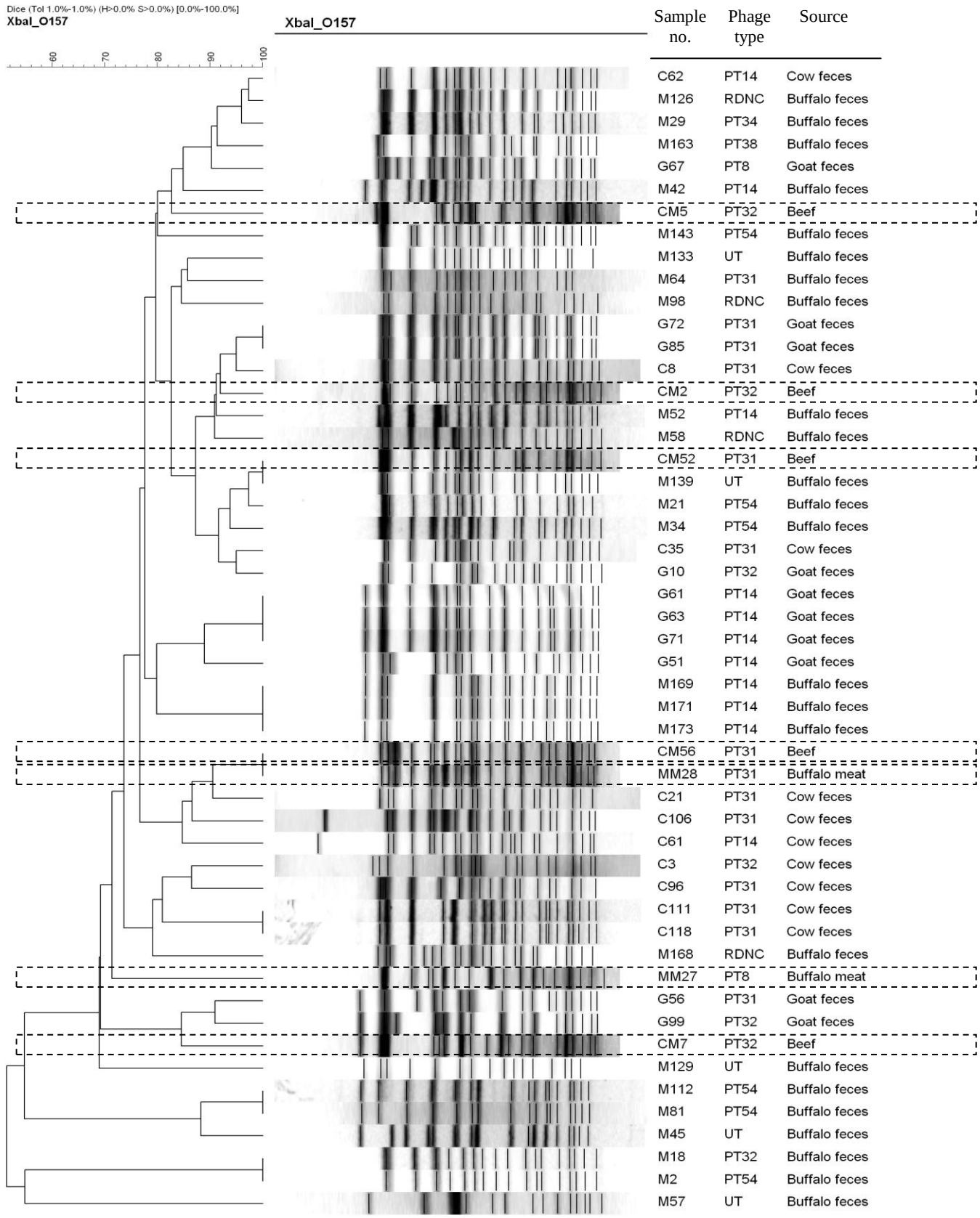
| *Sources (n = 213)* | *No. of PCR positive (%)* | | | | *No. of Isolates (%)* | | |
|---|---|---|---|---|---|---|---|
| | *Only $stx_1$* | *Only $stx_2$* | *$stx_{1+2}$* | *Total* | *E. coli O157*[a] | *Non-O157* | *Total* |
| Meat (90) | 1 (1.1) | 9 (10.0) | 52 (57.8) | 62 (68.9) | 7 (7.8) | 0 | 7 (7.8) |
| Buffalo (30) | 0 | 2 (6.7) | 19 (63.3) | 21 (70) | 2 (6.7) | 0 | 2 (6.7) |
| Beef (60) | 1 (1.7) | 7 (11.7) | 33 (55) | 41 (68.3) | 5 (8.3) | 0 | 5 (8.3) |
| Raw milk (20) | 1 (5) | 1 (5) | 0 | 2 (10) | 0 | 0 | 0 |
| Fresh juices (103) | 2 (1.9) | 4 (3.9) | 2 (1.9) | 8 (7.8) | 0 | 0 | 0 |
| Mixed herbal (49) | 2 (4) | 2 (4) | 2 (4) | 6 (12) | 0 | 0 | 0 |
| Sugarcane (40) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Wood apple (7) | 0 | 2 (28.6) | 0 (0) | 2 (28.6) | 0 | 0 | 0 |
| Others (7) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] By using immunomagnetic separation.

## Legend to figure

**FIG. 1.** Dendrogram generated by Bionumeric software, showing distance calculated by the dice similarity index of PFGE *Xba*I profiles for STEC O157 isolates isolated from raw meats ($n$ = 7) and animal feces ($n$ = 44). Box with dotted line indicated profiles of raw meat isolates. The degree of similarity (%) is shown on the scale.

1 **FIG.1.**


Dice (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
XbaI_O157
60
70
80
90
100
XbaI_O157
Sample no.
Phage type
Source
C62 PT14 Cow feces
M126 RDNC Buffalo feces
M29 PT34 Buffalo feces
M163 PT38 Buffalo feces
G67 PT8 Goat feces
M42 PT14 Buffalo feces
CM5 PT32 Beef
M143 PT54 Buffalo feces
M133 UT Buffalo feces
M64 PT31 Buffalo feces
M98 RDNC Buffalo feces
G72 PT31 Goat feces
G85 PT31 Goat feces
C8 PT31 Cow feces
CM2 PT32 Beef
M52 PT14 Buffalo feces
M58 RDNC Buffalo feces
CM52 PT31 Beef
M139 UT Buffalo feces
M21 PT54 Buffalo feces
M34 PT54 Buffalo feces
C35 PT31 Cow feces
G10 PT32 Goat feces
G61 PT14 Goat feces
G63 PT14 Goat feces
G71 PT14 Goat feces
G51 PT14 Goat feces
M169 PT14 Buffalo feces
M171 PT14 Buffalo feces
M173 PT14 Buffalo feces
CM56 PT31 Beef
MM28 PT31 Buffalo meat
C21 PT31 Cow feces
C106 PT31 Cow feces
C61 PT14 Cow feces
C3 PT32 Cow feces
C96 PT31 Cow feces
C111 PT31 Cow feces
C118 PT31 Cow feces
M168 RDNC Buffalo feces
MM27 PT8 Buffalo meat
G56 PT31 Goat feces
G99 PT32 Goat feces
CM7 PT32 Beef
M129 UT Buffalo feces
M112 PT54 Buffalo feces
M81 PT54 Buffalo feces
M45 UT Buffalo feces
M18 PT32 Buffalo feces
M2 PT54 Buffalo feces
M57 UT Buffalo feces